Neuroendocrine Stress Response in Female and Male Youths With Conduct Disorder and Associations With Early Adversity

Running head: Stress response in conduct disorder

Anka Bernhard, PhD, Katharina Ackermann, PhD, Anne Martinelli, PhD, Chiocchetti, Andreas G., PhD, Leonora Vllasaliu, PhD, Karen Gonzalez-Madruga, PhD, Molly Batchelor, BSc, Nora Maria Raschle, PhD, Helena Oldenhof, MSc, Lucres Jansen, PhD, Gregor Kohls, PhD, Kerstin Konrad, PhD, Arne Popma, PhD, Christina Stadler, PhD, Graeme Fairchild, PhD, & Christine M. Freitag, MD, PhD (Habilitation)

Authors' affiliations: Drs. Bernhard, Ackermann, Martinelli, Chiocchetti and Vllasaliu, and Prof. Freitag are with the University Hospital and Goethe University Frankfurt am Main, Germany. Dr. Ackermann is also with the Universität Hamburg, Germany. Dr. Martinelli is also with the Fresenius University of Applied Sciences Frankfurt am Main, Germany. Dr. Gonzalez-Madruga is with Middlesex University, UK. Ms. Batchelor is with the University of Southampton, UK. Profs. Raschle and Stadler are with the Psychiatric University Clinic and University of Basel, Switzerland. Prof. Raschle is also with the Jacobs Center for Productive Youth Development at the University of Zurich, Switzerland. Prof. Popma, Dr. Jansen and Ms. Oldenhof are with the Vrije Universiteit Amsterdam, The Netherlands. Dr. Kohls and Prof. Konrad are with the University Hospital RWTH Aachen, Germany. Prof. Konrad is also with the RWTH Aachen & Research Centre Juelich, Germany. Dr. Kohls is also with the Faculty of Medicine, TU Dresden, Germany. Dr. Fairchild is with the University of Bath, UK.

Correspondence to: Anka Bernhard, PhD, Department of Child and Adolescent Psychiatry, Psychosomatics and Psychotherapy, University Hospital Frankfurt, Goethe University Frankfurt am Main, Deutschordenstraße 50, D-60528 Frankfurt am Main, Germany; e-mail: anka.bernhard@kgu.de.

Acknowledgements: This study was funded by the European Commission's Seventh Framework Program (FP7 grant no. 602407; FemNAT-CD, coordinator Prof. Christine M. Freitag). The authors sincerely thank all participants and their parents/caregivers for participating, and Prof. Clemens Kirschbaum, TU Dresden, Germany, for his scientific advice.

Presentation information: Preliminary data from this study was presented at the Society of Biological Psychiatry's 72th Meeting; May 18-20, 2017; San Diego, USA and the 13th World Congress of Biological Psychiatry; June 18-22, 2017; Copenhagen, Denmark and the European Association for Forensic Child & Adolescent Psychiatry, Psychology & other involved professions' 6th Meeting; June 20-22, 2018; Venice, Italy and the International Society for Research on Aggression's 23th Meeting; July 10-14, 2018; Paris, France.

Disclosure: Prof. Konrad has received speaker fees from Shire Pharmaceuticals and Medice and receives royalties for books. Prof. Stadler receives royalties for a book on aggression. Prof. Freitag receives royalties for books on Autism Spectrum Disorder, ADHD, and depressive disorder. Drs. Bernhard, Ackermann, Martinelli, Chiocchetti, Vllasaliu, Gonzalez-Madruga, Raschle, Jansen, Kohls, Popma and Fairchild, and Mss. Batchelor and Oldenhof report no biomedical financial interests or potential conflict of interest.

Abstract

Objective: Conduct disorder (CD) involves aggressive and antisocial behavior and is associated with blunted cortisol stress response in male youths. Far less is known about cortisol stress responsivity in female youths with CD or other neuroendocrine responses in both sexes. Although CD is linked to early adversity, the possibility that neuroendocrine alterations may mediate the relationship between early adversity and CD has not been systematically investigated.

Method: Within the European FemNAT-CD multi-site study, salivary cortisol, testosterone, the testosterone/cortisol ratio, oxytocin, and psychological stress response to a standardized psychosocial stress test (the Trier Social Stress Test) together with common pre- and postnatal environmental risk factors were investigated in 130 pubertal youths with CD (63% female, 9-18 years) and 160 sex-, age-, and puberty-matched healthy controls (HCs).

Results: The TSST induced psychological stress in both CD and HCs. In contrast, female and male youths with CD showed blunted cortisol, testosterone, oxytocin, and testosterone/cortisol stress responses compared to HCs. These blunted stress responses partly mediated the relationship between environmental risk factors and CD.

Conclusion: Findings from this unique sample, including many female youths with CD, provide evidence for a widespread attenuated stress responsivity of not only stress hormones, but also sex hormones and neuropeptides in CD and its subgroups (e.g., with limited prosocial emotions). Results are the first to demonstrate blunted neuroendocrine stress responses in both female and male youths with CD. Early adversity may alter neuroendocrine stress responsivity. Biological mechanisms should be investigated further to pave the way for personalized intervention, thereby improving treatments for CD.

Key words: conduct disorder, stress response, cortisol, testosterone, oxytocin.

Introduction

Conduct disorder (CD) is characterized by aggressive, antisocial, and rule-breaking behavior, with most seriously affected individuals characterized by limited prosocial emotions.[1] CD is one of the most impairing, but also least studied mental disorders in childhood and adolescence, particularly in female individuals.[2] While prevalence rates increased in past decades, studies on female youths with CD remain scarce.[3] A growing body of evidence indicates that CD is associated with neurobiological alterations, such as changes in brain, autonomic nervous system, or neuroendocrine functioning.

One of the major neurobiological characteristics of CD is altered hypothalamic-pituitary-adrenal (HPA)-axis activity,[4] which is essential for responding to and regulating the impact of stress. During stress, the hypothalamus releases corticotropin-releasing hormone, triggering adrenocorticotropin release from the pituitary. Adrenocorticotropin stimulates the adrenal gland to release cortisol into the bloodstream.[5] Salivary cortisol serves as a widely-used peripheral marker of HPA-axis functioning. Lower salivary cortisol responses to public-speaking or anger-provocation tasks have been consistently reported in male youths with CD compared to healthy controls (HCs).[6] Importantly, it is unknown whether female youths with CD show similarly attenuated HPA-axis stress responsivity, although lower morning cortisol levels were reported in girls with CD compared with HCs.[7]

Previous studies on neuroendocrine stress responsivity in CD focused on the HPA-axis, mainly by assessing cortisol. This is surprising given the complex interplay between major neuroendocrine axes, e.g., the HPA-axis and the hypothalamic-pituitary-gonadal (HPG)-axis.[5] During stress responding, the HPA- and HPG-axes are assumed to work in a coordinated manner to enable successful stress-regulation.[8] Previous work on the HPG-axis focused on testosterone stress response, a predominantly male sex hormone. Yet, although concentrations

are lower than in male individuals, testosterone is also an essential part of the female neuroendocrine system. Sex hormones have not yet been assessed under stress in youths with CD. It was suggested that low basal cortisol in the presence of high testosterone increases risk for aggression (dual-hormone-hypothesis), although this has been questioned recently.[9-10] For stress response, interactions between cortisol and testosterone have not been investigated in CD. In addition to the HPG-axis, neuropeptides (e.g., oxytocin) influence HPA-axis responsivity. Oxytocin inhibits HPA-axis activity, evoking stress-protective and anxiolytic effects.[11] Most stressors stimulating HPA-axis activity also activate oxytocin. Peripheral salivary cortisol and oxytocin are assumed to be co-activated under stress, with oxytocin preceding cortisol release.[12-13] Despite its possible role in stress-regulation, oxytocin stress response has not been investigated in CD though recent reports of salivary oxytocin stress responsivity in healthy youth encourage its assessment in CD.[13] Given the simultaneous activation of oxytocin, the HPA- and HPG-axis in response to stress, it is important to apply a multi-system approach to understand the overarching neurobiology of stress-regulation in CD.

Additionally, given that CD is strongly associated with early adversity,[14] findings of blunted neuroendocrine stress responsivity may be influenced by exposure to prenatal (e.g., maternal smoking) or postnatal risk factors (e.g., exposure to trauma or familial conflicts). Such early adversity has been associated with altered HPA-axis, HPG-axis and neuropeptide functioning.[15–17] Attenuated neuroendocrine stress responsivity may represent a functional adaptation to early-life adversity.[18] A recent review highlighted the importance of considering early adversity in neuroendocrine research on aggression.[19] While no previous study investigated whether neuroendocrine stress response mediates the relation between early adversity and CD, such work is strongly warranted to understand the neurobiological mechanisms that may underpin the adversity-CD association.

Overall, an appropriate response to an acute stressor is crucial for an individual's successful stress-regulation, with alterations of neuroendocrine stress responsivity constituting a risk factor for psychiatric disorders. Likewise, altered stress responsivity has been reported in CD. Critically, all previous research on neuroendocrine stress responsivity was limited to boys with CD, thus almost nothing is known about stress responsivity in girls with CD. Furthermore, previous neuroendocrine research focused on HPA-axis stress responsivity and neglected other related neuroendocrine axes, as well as the possibility that associations between early adversity and CD may be mediated by neuroendocrine alterations. To address these research gaps and better understand the potential role of altered stress regulation in both girls and boys with CD, we comprehensively investigated multiple neuroendocrine axes (HPA-axis: cortisol, HPG-axis: testosterone, neuropeptides: oxytocin) in response to a standardized and validated psychosocial stress test in female and male youths with CD compared to sex-matched HCs within a European multi-site study. Given previous evidence of cortisol hypo-reactivity in girls and boys with CD,[4,7] and coordinated HPA-axis, HPG-axis and neuropeptide stress responsivity,[8,11] we hypothesized an overall attenuated neuroendocrine response in female and male youths with CD compared to sex-matched HCs. Additionally, we explored whether neuroendocrine stress response mediated the relationship between common pre- and postnatal risk factors and CD, to generate evidence which could provide a foundation for studies testing specific hypotheses.

Method

Participants

This study included 130 pubertal youths (9-18 years) with CD (82 female participants) and 160 HCs (104 female participants) from the European FemNAT-CD-project.[3] Data were collected between 2014-2017 across five European sites (Germany, Switzerland, the Netherlands, UK; see Table S1, available online). Most participants were born in the country of assessment (Mean % [SD]: youths 95.04 [2.96], parents 75.11 [8.94]) and were of white European ancestry. Local ethical committees at each site approved the study. Participants were recruited from clinics, youth offending services, schools, and the community (e.g., newspaper adverts, social media). Written informed consent was obtained from all participants and/or their legal guardians after detailed study explanation. Exclusion criteria included IQ<70, pre-pubertal status, pregnancy, last menstruation>6 months ago, history of neurological disorder, traumatic brain injury, schizophrenia, autism spectrum disorder, or current mania or bipolar disorder. Youths in the CD group fulfilled DSM-IV-TR criteria for current CD (CD symptoms range, Mean [SD]: CD 3-12, 5.25 [2.18], HCs: 0-1, 0.08 [0.26]).[20] HCs were omitted if they had any current DSM-IV-TR disorders or a history of disruptive behavior- or attention-deficit/hyperactivity disorder (ADHD).

Procedures

As in earlier FemNAT-CD publications,[21–23] current and lifetime psychiatric disorders were assessed using the Kiddie-Schedule for Affective Disorders and Schizophrenia-Present and Lifetime version (K-SADS-PL), IQ using the Wechsler Intelligence Scales, and pubertal status using the Pubertal Development Scale. This is a five-level categorical (pre-/early-/mid-/late-/post-pubertal) self-report measure on pubertal growth (e.g., changes in body hair, voice, or breast development; menarche) ranging from pre-pubertal (no changes) to post-pubertal status (all changes completed). Participants and/or parents/caregivers reported on four widely

replicated pre- and postnatal risk factors for CD: prenatal smoking and violence exposure, adverse family situation (familial disharmony and isolation), and the number of traumatic events experienced from the posttraumatic stress disorder (PTSD) K-SADS-PL section. To assess the DSM-5 CD limited prosocial emotions (LPE) specifier,[1] the unemotionality, callousness and remorselessness subscales of the self-report Youth Psychopathic traits Inventory were used, in line with earlier reports.[24-25] For details of all measures, see Supplement 1, available online.

Psychoneuroendocrine assessment

For details, see Supplement 1, available online. In brief, stress responsivity (see Figure 1) was assessed applying the Trier Social Stress Test (TSST),[26] a widely-used, standardized method for inducing psychosocial stress in laboratory settings.[27] The TSST involves completing public speaking and mental arithmetic tasks in front of a panel of strangers. Standardized operating procedures were used at each site with adherence controlled by external monitors. To confirm stress induction, participants rated their stress feelings ("Do you feel stressed?") eight times from baseline to +55 minutes after stress termination using a Visual Analog Scale (range 0-10).[28] Saliva samples were collected with Salivettes (Sarstedt, Germany) at baseline, +1, +10, +25, +40, and +55 minutes after stress termination. Samples were stored at -20°C until analysis.

[Figure 1]

Levels of salivary cortisol (nmol/L) at baseline, +10, +25, +40 and +55 minutes, and testosterone (pg/ml) at baseline, +10 and +55 minutes were analyzed by daacro's Saliva Lab (Trier, Germany; https://daacro.de/en/saliva-lab-trier/) employing an enzyme immunoassay kit (Salimetrics, LLC, USA) formatted to minimize cross-reactivity for related steroids. The corresponding inter- and intra-assay coefficients of variation were in the commonly accepted range (<15% and 10%, respectively).[29] After cortisol and testosterone analyses, the baseline, +1 and +10 minutes Salivettes were transferred to RIAgnosis (Sinzing, Germany) for

quantification of salivary oxytocin (pg/ml) by radioimmunoassay. The detection limit was in the 0.1-0.5 pg/sample range. Intra- and inter-assay coefficients of variation were <10%. Oxytocin was measured in a sub-sample of 170 participants (see Table S1, available online), including 80 youths with CD (45 female participants) and 90 HCs (49 female participants). Collection of samples at the +1 minute timepoint (optimal for oxytocin) commenced around one year into the study, after validation of salivary oxytocin stress responsiveness in youths.[13] Different timepoints for cortisol, testosterone and oxytocin analyses were based on reported distinct reactivity patterns.[12,13,27]

Statistical Analyses

Statistical analyses were performed using SPSS v24 (IBM Corp., Armonk, NY) and R v4.0.4. Significance levels of all tests were set at $p<0.05$ (two-tailed). Demographic and clinical characteristics were compared using univariate analyses of variance or Chi-Squared-tests. Correlations between the area under the curve with respect to increase (AUC_I) of the different psychoneuroendocrine measures,[30] as well as of these with CD age-of-onset (age range) and CD severity (CD symptoms range) were calculated using Spearman-correlations.

Psychoneuroendocrine stress responses were analyzed using five separate hierarchical linear models (HLM; R-function lmer(); lme4 package). The five dependent variables were psychological stress, cortisol, testosterone, testosterone/cortisol ratio, and oxytocin, with independent variables of group (CD versus HCs) and sex (female versus male) interacting with a linear (polynomial 1) and quadratic (polynomial 2) time effect. Next to site and individual participant as random effects, psychological stress analyses controlled for age, and neuroendocrine analyses for age, current smoking, body mass index (BMI), TSST start time, and medication (incl. contraceptive) as fixed effects. Neuroendocrine measures were log-transformed to normalize their distribution. Dimensional covariates were mean-centered. Our sample size was sufficient to detect a small (N=290 overall sample) to moderate (n=170

oxytocin sample) effect for each predictor of interest (main and interaction effects of group, sex, and both time factors) with a power of 80% and a two-sided significance level of 5% (G*Power 3.1.9.2; see Supplement 1, available online). To follow up possible interactions of sex, group, and any time factor, each HLM was repeated in female and male youths separately. Sensitivity analyses were performed repeating each HLM including the potential confounding factor in separate additional models: IQ, parental educational status, pubertal status, and psychiatric comorbidities [ADHD, major depressive disorder (MDD), PTSD, substance use disorder (SUD), anxiety disorders (ANX)]. Each HLM was also run with CD severity (indexed via CD symptoms) and the LPE specifier (CD+LPE, CD-LPE, HCs+LPE, HCs-LPE) replacing group (CD versus HCs). While the HLM approach has numerous strengths,[31] for easier comparison with previous work, repeated measures analyses of co-variance were also performed (see Supplement 1, available online).

To explore mediating effects of psychoneuroendocrine responses on associations between pre- or post-natal risk factors and CD status, mediation analyses (see Supplement 1, available online) were performed using the PROCESS macro (http://www.processmacro.org/). In brief, aggregated psychoneuroendocrine stress response measures were computed as mediators: the AUC_I, stress-increase (difference baseline-peak) and post-stress recovery (difference peak-lowest level after stress termination).[13] For each risk factor and possible mediator, a mediation analysis was run yielding direct, indirect, and total effects. Applying bootstrapping with 1000 resamples, percentile bootstrap confidence intervals (CIs) for the indirect effects were computed. The null hypothesis of no mediation effect was rejected if $0 \notin 95\%\,\text{CI}$.[32] As the mediation analyses investigated associations between risk factors and psychoneuroendocrine stress measures in an exploratory manner, results are presented descriptively without correction for multiple comparisons.

Results

Sample characteristics

Groups did not differ in sex, age, pubertal status, or TSST start time (see Table 1; data for oxytocin subsample in Table S2, available online). Participants with CD had higher BMIs, and rates of current smoking, medication use, +LPE specifier, risk factors, and psychiatric comorbidities, but lower IQ and parental educational status than HCs. Most female participants had started menstruating (CD 92%, HCs 79%) with comparable days since the last menstruation (Mean [SD]: CD 21.16 [18.22], HCs 20.09 [14.15], p=.71). Baseline psychological stress (mean [SD]: CD=1.48 [2.19], HCs=1.12 [1.89], p=.11), cortisol (nmol/L; mean [SD]: CD=3.03 [1.61], HCs=2.86 [1.93], p=.74), testosterone (pg/ml; mean [SD]: CD=47.31 [25.34], HCs=42.64 [24.78], p=.17), and oxytocin (pg/ml; mean [SD]: CD=1.24 [0.45], HCs=1.34 [0.65], p=.20) did not differ between groups.

[Table 1]

Correlations between psychoneuroendocrine stress responses

The AUC_I for psychological stress did not correlate with cortisol, testosterone, and oxytocin AUC_I, neither in the overall sample nor in male and female participants considered separately (for correlations see Table S3, available online). Cortisol and testosterone AUC_I correlated positively in the overall sample (r=.45, p<.001), in the CD (r=.36, p<.001) and HC groups (r=.43, p<.001), and in all sex-separated groups ($rs \geq .29$, ps<.05). Oxytocin AUC_I did not correlate with cortisol or testosterone AUC_I in any group.

CD severity correlated positively with psychological stress (r=.16, p=.01), and negatively with cortisol (r=-.37, p<.001) and testosterone (r=-.24, p<.001), but not oxytocin AUC_I in the overall (N=290) and female (n=186) samples. In the male sample (n=104), CD severity correlated negatively with cortisol (r=-.37, p<.001). CD age-of-onset did not correlate with any psychoneuroendocrine measure (see Table S4, available online).

Hierarchical linear models of psychoneuroendocrine stress response

The HLM results are shown in Table 2. Table S5, available online, and Figure 2 present original data; Figure S1, available online, displays log-transformed values.

Stress was successfully induced using the TSST as indicated by a significant linear main effect of time. No interactions of group, sex, and both time variables were observed, indicating a similar psychological stress response in female and male participants with and without CD.

The TSST induced a strong neuroendocrine stress response in HCs, but not in participants with CD for all neuroendocrine measures. Regarding linear change over time (polynomial 1), groups strongly differed in their cortisol and testosterone stress response, with HCs but not participants with CD showing a clear increase irrespective of sex (group-by-time, but no group-by-sex-by-time effect). For quadratic change over time (polynomial 2), a group-by-sex-by-time effect was found for cortisol and testosterone. Followed-up by sex-specific HLMs, a stronger cortisol group-by-time effect was found in female compared to male participants. The difference between female HC and CD participants for cortisol increase and decrease was greater than that between male HC and CD participants (i.e., female HC participants had the highest cortisol AUC_I). For testosterone, a quadratic group-by-time effect was observed in female, but not male participants. Curvilinear testosterone increase and decrease were stronger in female HC than female CD participants, while in male participants the group difference particularly for stress recovery was less evident (due to slower decrease in healthy male youths). For the testosterone/cortisol ratio, a group-by-time effect was found. The ratio remained stable in female and male participants with CD, showing a coordinated hypo-reactivity of both steroids (see Figure S1, available online). In healthy female and male youths, a curvilinear increase and decrease of the testosterone/cortisol ratio in response to stress was found. For oxytocin, the quadratic time effect differed between groups irrespective of sex showing oxytocin stress response across increase and decrease in HCs but not in individuals with CD. Classical rmANCOVAs showed similar results (see Table S6, available online).

[Table 2, Figure 2]

Sensitivity analyses

Repeating each HLM with IQ, pubertal status, or psychiatric comorbidity (ADHD, MDD, PTSD, SUD, or ANX) as an additional predictor did not change the reported effects for all psychoneuroendocrine measures. When including parental educational status, findings remained unchanged except for the group-by-time interaction for testosterone/cortisol ratio (*b*=-3.86, *SE*=2.69, *t*=-1.44, *p*=.15). When considering CD symptom severity instead of group in each HLM (see Table S7, available online), effects for psychological stress and cortisol remained comparable. For testosterone, the linear, while for oxytocin and the testosterone/cortisol ratio the quadratic group-by-time effect lost significance. Comparing participants with and without the LPE specifier (see Table S8 and Figure S2, available online), no time-course differences for psychological stress, cortisol, testosterone, the testosterone/cortisol ratio, and oxytocin were found between CD+LPE and CD-LPE. Only in the HC group, stronger cortisol responses were observed in HCs-LPE than HCs+LPE.

Exploratory mediation analyses

In additional exploratory analyses (see Figure 3; Table S9, available online), psychological stress AUC_I mediated the relation between adverse family situation and CD (5% of the total effect). Cortisol AUC_I mediated the relationship between prenatal violence exposure and adverse family situation with CD, explaining 7-10% of the total effect. Similar mediation effects were observed for cortisol increase (13-15% of the total effect). Cortisol recovery mediated the relationships between prenatal smoking, adverse family situation and trauma exposure with CD (13-16% of the total effect). Oxytocin increase mediated the relation between prenatal smoking and CD, and oxytocin recovery between prenatal smoking and violence with CD (10-13% of the total effect). No other significant mediating effects (including for testosterone) were found.

[Figure 3]

Discussion

This is the first and largest study to date which comprehensively examined whether female youths with CD show similarly attenuated HPA-axis stress response compared to male youths with CD within the context of an European multi-site study. It is also the first to simultaneously investigate other neuroendocrine axes. Our results replicate and considerably extend previous studies focusing on cortisol stress response in male individuals with CD by providing evidence for a diminished neuroendocrine stress responsivity of not only the HPA-axis, but also the HPG- and neuropeptide axes in CD. Critically, neuroendocrine stress hypo-reactivity was observed in both female and male youths with CD compared to healthy youths. Remarkably, neuroendocrine alterations were more pronounced in female than male participants with CD. Changes in psychoneuroendocrine stress responsivity mediated the relationship between pre- and postnatal risk factors and CD, suggesting exposure to early adversity may affect attenuated neuroendocrine stress reactivity as a biological marker of CD. Analyses considered highly relevant confounders such as current smoking, BMI, TSST start time, and medication use (incl. contraceptives). Controlling for IQ, parental educational status, pubertal status, and psychiatric comorbidities did not substantially affect results. The female CD and HC groups did not differ in menstrual cycle status with most girls being tested in the luteal phase, during which the neuroendocrine stress response is more comparable to male individuals.[33]

The TSST induced substantial increases in psychological stress in both female and male CD and HC participants. Consistent with previous studies, individuals with CD were similarly psychologically affected by stress as HCs,[4] with psychological and neuroendocrine stress being uncorrelated.[34-35] Thus, our finding of substantially altered neuroendocrine stress responsivity in individuals with CD compared to HCs can be considered as highly valid as the TSST was

effective in inducing feelings of stress. While HC youths showed clear cortisol, testosterone, and oxytocin responses to the TSST (comparable to previous reports in healthy youths),[8,13,27] a widespread attenuation of neuroendocrine stress responsivity was found in youths with CD. Consistent with earlier reports,[4] we observed blunted cortisol stress responsivity in male youths with CD. Importantly, our results also demonstrate attenuated cortisol stress response in female youths with CD. Notably, differences between CD and HC participants were even stronger in female than male youths for cortisol and testosterone stress responses. Female youths with CD seem to particularly differ in their neuroendocrine stress response from healthy female youths. While this may be in line with recent findings showing girls with CD to be more clinically impaired than boys,[22] results need to be replicated. As earlier work did not investigate female and male youths with CD in response to a standardized stress task, our findings are the first to indicate blunted HPA-axis activity as a distinct characteristic of CD independent of sex. Furthermore, our results substantially extend previous work limited to cortisol by showing comparable attenuations in testosterone and oxytocin stress response in both female and male youths with CD. Attenuated cortisol and oxytocin stress responses were not explained by psychiatric comorbidities (e.g., ADHD, PTSD, anxiety, depressive or substance use disorders). As reported earlier, CD age-of-onset did not influence results,[36] but with increased CD severity particularly cortisol stress responses were more strongly attenuated.[37-38] This points to a difference between CD as categorical diagnosis and CD symptom counts particularly for testosterone and oxytocin, which may be specifically relevant for severely affected youth meeting CD criteria. Moreover, neuroendocrine stress responses were similarly blunted in CD youths with and without LPE, contrary to notions that cortisol hypo-reactivity may be specific to the former subgroup.[39] Consistent with earlier reports,[24] 19% of our HC sample fulfilled criteria for the LPE specifier and this variable explained some variance in neuroendocrine responsivity in this group. Given the small sample size of these subgroups, future studies on neuroendocrine stress responsivity should investigate the influence of CD-specific traits (e.g.,

callous-unemotional-traits, proactive/reactive aggression),[40-41] applying dimensional rather than categorical approaches in female and male youths with CD, but also in population-based samples. Different developmental pathways, such as primary (driven by genetically-based deficits in emotion processing) and secondary (driven by experiences of adversity) variants of psychopathy/callous-unemotional-traits, and the critical role of trauma (e.g., comparing youth with/without trauma exposure) should also be considered.[42–44]

Our findings of attenuated steroid hormone and neuropeptide stress response suggest an overall altered neuroendocrine stress-regulation in female and male youths with CD. As reported recently,[8–10] and in contrast to the dual-hormone-hypothesis, our data support a coordinated HPA- and HPG-axis stress responsiveness. Individuals with higher HPA-axis reactivity showed higher HPG-axis and neuropeptide responses, and vice-versa. Coordination across HPA- and HPG-axes was particularly strong in individuals with CD (similarly blunted responses). In HCs, both stress and sex hormones increased and then decreased in response to stress, though cortisol showed a stronger reactivity than testosterone in accordance with the central role of the HPA-axis in stress regulation. Coordinated neuroendocrine stress responsivity may be strongly functional, both for short-term adaptive (e.g., cortisol activating energy resources, testosterone improving performance),[8] and long-term chronic stress regulation. An overall blunted neuroendocrine stress response in CD may result from a continuing regulatory process (e.g., in severely stressful environments) during which an initially responsive system becomes unresponsive to avoid allostatic overload.[18]

One major contributing factor underlying stress hypo-reactivity in CD may be early adversity exposure, reported to attenuate neuroendocrine stress responsivity,[15–17] and strongly associated with CD.[14] Exploring the influence of psychoneuroendocrine stress responses on the relation between pre- and post-natal risk factors and CD, mediation effects were found for psychological stress response, cortisol, and oxytocin (all particularly relevant for stress-

regulation). Exposure to prenatal smoking/violence, adverse family situation, and trauma not only predicted CD, but also attenuated cortisol and oxytocin accompanied by higher psychological stress response. These alterations, in turn, predicted CD. Our results indicate psychoneuroendocrine alterations in CD to be associated with early adversity accordingly with similar neurobiological mediation effects of structural brain alterations.[45] Attenuated neuroendocrine stress responsivity may constitute an underlying mechanism by which environmental risks contribute to CD risk, driven by an early adaptation to adversity.[18] Given the exploratory design, replication is needed, at best longitudinal studies investigating the underlying biological and neural mechanisms during individual development.

Considering impaired psychoneuroendocrine stress-regulation and associated risk factors when treating CD may help to improve individuals' stress coping strategies, thereby reducing CD-related behaviors. Blunted cortisol stress responsivity predicted poorer treatment outcomes in youths with disruptive behavior disorders.[46] Personalized treatments based on an individual's neurobiological characteristics may improve successful interventions for CD, e.g., by implementing additional pharmacological treatments in at-risk individuals. However, effects of personalized CD interventions and pharmacological treatments to normalize neuroendocrine hypo-reactivity are yet to be studied.

This study has several strengths. We included a large and representative sample of female and male youths with and without CD, recruited across multiple European countries, the groups being sex-, age-, and puberty-matched, allowing sex-specific analyses. Additional sensitivity analyses confirmed the stability of the results. Participants with CD and HCs were diagnosed using standardized semi-structured diagnostic interviews based on DSM-IV-TR criteria, with the participants and their parents/caregivers interviewed separately. Regular monitoring during the FemNAT-CD-project ensured adherence to all procedures (e.g., TSST, saliva sampling/shipping). Our study is the first to investigate psychoneuroendocrine stress

response in girls compared to boys with CD, applying a standardized, validated and widely-used laboratory psychosocial stress induction procedure, the TSST,[26-27] in comparison to a healthy control group whose neuroendocrine (and psychological) stress responsivity demonstrated successful stress induction. While previous work in boys with CD focused on cortisol reactivity alone, we also assessed two hitherto unstudied neuroendocrine axes in response to stress: the HPG-axis (testosterone) and neuropeptides (oxytocin). Importantly, we also controlled for major confounding variables (e.g., smoking, BMI, TSST start time, medication), and investigated the impact of CD-specific clinical characteristics (e.g., age of onset, CD severity, LPE specifier) on psychoneuroendocrine stress responsivity. Finally, this is the first study to explore whether neuroendocrine alterations mediate the effects of early adversity for CD.

Our study also has several limitations. First, the five European sites contributed different sample sizes according to site-specific recruitment possibilities. For comparability, standardized operating procedures were used at each site during all assessments with adherence controlled by external monitors. Site was also included as random effect in the analyses. Second, the age range was relatively large, but groups matched, and age included as fixed effect. Third, sample of oxytocin stress responsivity was smaller than of cortisol and testosterone measures, but still relatively large (n=170) compared to previous studies. We assessed salivary oxytocin, which has been a topic of controversy, particularly due to its assumed short-life, molecular weight and impurity.[47] While steroid hormones can pass the blood-brain-barrier, non-invasive peripheral salivary assessment may not fully capture central oxytocin release. However, more recently, salivary oxytocin has been consistently reported as a valid, non-stressful (particularly relevant for stress response research) assessment in youths and adults,[12-13] correlating with its central concentrations.[48] The range of oxytocin values in our study was also in line with previous work.[12-13,47] Fourth, storage of saliva samples at -20°C was reported

as less optimal than -80°C.[49] As storage was akin across groups, possible degradation by storage temperature may only have influenced absolute, rather than relative concentrations. Fifth, information on menstrual cycle status was assessed via self-reported dates of last menstruation. Future studies should consider recent recommendations when assessing and controlling for menstrual cycle stage[50]. Sixth, the uneven distribution of comorbid psychiatric disorders between groups and sexes limited its consideration. HC participants were not free of lifetime, e.g., internalizing disorders, and sensitivity analyses showed no substantial influence of psychiatric comorbidities on psychoneuroendocrine stress responses. To fully investigate possible influences of psychiatric comorbidities, future studies should include participants with other common comorbid disorders. Finally, only a small number of retrospectively assessed pre- and postnatal risk factors for CD were included, predominantly relying on parent-report data. Mediation analyses were performed in an exploratory fashion without hypotheses and in a descriptive manner, thus no correction for multiple comparisons was applied. Further studies are needed to replicate and extend our preliminary findings; ideally, with a prospective longitudinal design to thoroughly investigate developmental pathways and temporal relationships between risk factors and neuroendocrine alterations.

In conclusion, this work substantially extends current knowledge about stress responsivity in CD beyond male individuals. It is the first study to provide compelling evidence for attenuated stress response in female individuals with CD, in addition to their male counterparts, based on a European multi-site sample. Our study not only demonstrates reduced cortisol stress responsivity, but also testosterone and oxytocin hypo-reactivity, in both female and male youths with CD compared to healthy youths. While psychological stress responsivity was comparable to healthy youths, our results indicate a substantially altered neuroendocrine stress response in CD in both sexes. Our findings suggested that female youths with CD may show more pronounced cortisol and testosterone alterations than male youths with CD (in

comparison with sex-matched HCs). Furthermore, we observed similar neuroendocrine hypo-reactivity across different clinical characteristics of CD such as with and without the LPE specifier. Results were unrelated to potentially confounding factors such as smoking, BMI, TSST start time, medication (incl. contraceptives), IQ, pubertal status, parental educational status, or site. Our findings further suggest neuroendocrine alterations as a possible underlying mechanism through which early adversity may contribute to risk for CD. Considering neurobiological characteristics and associated environmental risks in personalizing an individual's treatment may help to improve future interventions for CD.

References

1. American Psychiatric Association. *Diagnostic and Statistical Manual of Mental Disorders, Fitfth Edition (DSM-5)*. American Psychiatric Association; 2013.
2. Fairchild G, Hawes DJ, Frick PJ, et al. Conduct disorder. *Nat Rev Dis Prim*. 2019;5(43):1-25. doi:10.1038/s41572-019-0095-y
3. Freitag CM, Konrad K, Stadler C, et al. Conduct disorder in adolescent females-current state of research and study design of the Fem-NAT-CD consortium. *Eur Child Adolesc Psychiatry*. 2018;27:1077-1093. doi:10.1007/s00787-018-1172-6
4. Fairchild G, Baker E, Eaton S. Hypothalamic-Pituitary-Adrenal Axis Function in Children and Adults with Severe Antisocial Behavior and the Impact of Early Adversity. *Curr Psychiatry Rep*. 2018;20(84):1-9. doi:10.1007/s12192-010-0223-9
5. Oyola MG, Handa RJ. Hypothalamic–pituitary–adrenal and hypothalamic–pituitary–gonadal axes: sex differences in regulation of stress responsivity. *Stress*. 2017;20(5):476-494. doi:10.1080/10253890.2017.1369523.Hypothalamic
6. Bernhard A, Mayer JS, Fann N, Freitag CM. Cortisol response to acute psychosocial stress in ADHD compared to conduct disorder and major depressive disorder: A systematic review. *Neurosci Biobehav Rev*. 2021;127:899-916. doi:10.1016/j.neubiorev.2021.06.005
7. Pajer K, Gardner W, Rubin RT, Perel J, Neal S. Decreased cortisol levels in adolescent girls with conduct disorder. *Arch Gen Psychiatry*. 2001;58(3):297-302. doi:10.1001/archpsyc.58.3.297
8. Turan B, Tackett JL, Lechtreck MT, Browning WR. Coordination of the cortisol and testosterone responses: A dual axis approach to understanding the response to social status threats. *Psychoneuroendocrinology*. 2015;62:59-68. doi:10.1016/j.psyneuen.2015.07.166

9. Grebe NM, Del M, Emery M, et al. Testosterone, cortisol, and status-striving personality features: A review and empirical evaluation of the Dual Hormone hypothesis. *Horm Behav*. 2019;109:25-37. doi:10.1016/j.yhbeh.2019.01.006

10. Dekkers TJ, van Rentergem JAA, Meijer B, Popma A, Wagemaker E, Huizenga HM. A meta-analytical evaluation of the dual-hormone hypothesis: Does cortisol moderate the relationship between testosterone and status, dominance, risk taking, aggression, and psychopathy? *Neurosci Biobehav Rev*. 2019;96:250-271. doi:10.1016/j.neubiorev.2018.12.004

11. Torner L, Plotsky PM, Neumann ID, de Jong TR. Forced swimming-induced oxytocin release into blood and brain: Effects of adrenalectomy and corticosterone treatment. *Psychoneuroendocrinology*. 2017;77:165-174. doi:10.1016/j.psyneuen.2016.12.006

12. de Jong TR, Menon R, Bludau A, et al. Salivary oxytocin concentrations in response to running, sexual self-stimulation, breastfeeding and the TSST: The Regensburg Oxytocin Challenge (ROC) study. *Psychoneuroendocrinology*. 2015;62:381-388. doi:10.1016/j.psyneuen.2015.08.027

13. Bernhard A, van der Merwe C, Ackermann K, Martinelli A, Neumann ID, Freitag CM. Adolescent oxytocin response to stress and its behavioral and endocrine correlates. *Horm Behav*. 2018;105:157-165. doi:10.1016/j.yhbeh.2018.08.010

14. Bernhard A, Martinelli A, Ackermann K, Saure D, Freitag CM. Association of trauma, Posttraumatic Stress Disorder and Conduct Disorder: A systematic review and meta-analysis. *Neurosci Biobehav Rev*. 2018;91:153-169. doi:10.1016/j.neubiorev.2016.12.019

15. Koss KJ, Gunnar MR. Early adversity, the hypothalamic–pituitary–adrenocortical axis, and child psychopathology. *J Child Psychol Psychiatr*. 2018;59(4):327-346. doi:10.1111/jcpp.12784

16. Bunea IM, Szentágotai-Tatar A, Miu AC. Early-life adversity and cortisol response to

social stress: a meta-analysis. *Transl Psychiatry*. 2017;7(1274):1-8. doi:10.1038/s41398-017-0032-3

17. Tobon AL, Newport DJ, Nemeroff CB. The Role of Oxytocin in Early Life Adversity and Later Psychopathology: a Review of Preclinical and Clinical Studies. *Curr Treat Options Psych*. 2018;5:401-415. doi:10.1007/s40501-018-0158-9
18. Del Giudice M, Ellis BJ, Shirtcliff EA. The Adaptive Calibration Model of stress responsivity. *Neurosci Biobehav Rev*. 2011;35(7):1562-1592. doi:10.1016/j.neubiorev.2010.11.007.The
19. Fragkaki I, Cima M, Granic I. The role of trauma in the hormonal interplay of cortisol, testosterone, and oxytocin in adolescent aggression. *Psychoneuroendocrinology*. 2018;88:24-37. doi:10.1016/j.psyneuen.2017.11.005
20. American Psychiatric Association. *Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition (DSM-IV-TR)*. American Psychiatric Association; 2000.
21. Kohls G, Baumann S, Gundlach M, et al. Investigating Sex Differences in Emotion Recognition, Learning, and Regulation Among Youths With Conduct Disorder. *J Am Acad Child Adolesc Psychiatry*. 2020;59(2):263-273. doi:10.1016/j.jaac.2019.04.003
22. Konrad K, Kohls G, Baumann S, et al. Sex differences in psychiatric comorbidity and clinical presentation in youths with conduct disorder. *J Child Psychol Psychiatry*. Published online 2021. doi:10.1111/jcpp.13428
23. Bernhard A, Kirchner M, Martinelli A, et al. Sex-specific associations of basal steroid hormones and neuropeptides with Conduct Disorder and neuroendocrine mediation of environmental risk. *Eur Neuropsychopharmacol*. 2021;49:40-53. doi:10.1016/j.euroneuro.2021.03.016
24. Kohls G, Fairchild G, Bernhard A, et al. Neuropsychological Subgroups of Emotion Processing in Youths With Conduct Disorder. *Front psychiatry*. 2020;11:585052. doi:10.3389/fpsyt.2020.585052

25. Jambroes T, Jansen LMC, Vermeiren RRJM, Doreleijers TAH, Colins OF, Popma A. The clinical usefulness of the new LPE specifier for subtyping adolescents with conduct disorder in the DSM 5. *Eur Child Adolesc Psychiatry*. 2016;25(8):891-902. doi:10.1007/s00787-015-0812-3

26. Kirschbaum C, Pirke KM, Hellhammer DH. The 'Trier Social Stress Test'-A Tool for Investigating Psychobiological Stress Responses in a Laboratory Setting. *Neuropsychobiology*. 1993;28:76-81. doi:10.1159/000119004

27. Allen AP, Kennedy PJ, Cryan JF, Dinan TG, Clarke G. Biological and psychological markers of stress in humans: Focus on the Trier Social Stress Test. *Neurosci Biobehav Rev*. 2014;38:94-124. doi:10.1016/j.neubiorev.2013.11.005

28. Hellhammer J, Schubert M. The physiological response to Trier Social Stress Test relates to subjective measures of stress during but not before or after the test. *Psychoneuroendocrinology*. 2012;37(1):119-124. doi:10.1016/j.psyneuen.2011.05.012

29. Salimetrics. Calculating inter-and intra-assay-coefficients of variability. *https://salimetrics.com/calculating-inter-and-intra-assay-coefficients-of-variability/*. Published online 2021.

30. Pruessner JC, Kirschbaum C, Meinlschmid G, Hellhammer DH. Two formulas for computation of the area under the curve represent measures of total hormone concentration versus time-dependent change. *Psychoneuroendocrinology*. 2003;28(7):916-931. doi:10.1016/S0306-4530(02)00108-7

31. Dijkers MP. Chasing Change: Repeated-Measures Analysis of Variance Is So Yesterday! *Arch Phys Med Rehabil*. 2013;94:597-599. doi:10.1016/j.apmr.2012.11.018

32. Preacher KJ, Hayes AF. Asymptotic and resampling strategies for assessing and comparing indirect effects in multiple mediator models. *Behav Res Methods*. 2008;40(3):879-891. doi:10.3758/BRM.40.3.879

33. Kajantie E, Phillips DIW. The effects of sex and hormonal status on the physiological

response to acute psychosocial stress. *Psychoneuroendocrinology*. 2006;31(2):151-178. doi:10.1016/j.psyneuen.2005.07.002

34. Campbell J, Ehlert U. Acute psychosocial stress: Does the emotional stress response correspond with physiological responses? *Psychoneuroendocrinology*. 2012;37(8):1111-1134. doi:10.1016/j.psyneuen.2011.12.010

35. Joseph NT, Jiang Y, Zilioli S. Momentary emotions and salivary cortisol: A systematic review and meta-analysis of ecological momentary assessment studies. *Neurosci Biobehav Rev*. 2021;125:365-379. doi:10.1016/j.neubiorev.2021.02.042

36. Fairchild G, Goozen SHM Van, Stollery SJ, et al. Cortisol Diurnal Rhythm and Stress Reactivity in Male Adolescents with Early-Onset or Adolescence-Onset Conduct Disorder. *Biol Psychiatry*. 2008;64(7):599-606. doi:10.1016/j.biopsych.2008.05.022

37. de Vries-Bouw M, Jansen L, Vermeiren R, Doreleijers T, van de Ven P, Popma A. Concurrent attenuated reactivity of alpha-amylase and cortisol is related to disruptive behavior in male adolescents. *Horm Behav*. 2012;62:77-85. doi:10.1016/j.yhbeh.2012.05.002

38. Schoorl J, Rijn S van, Wied M de, van Goozen S, Swaab H. The role of anxiety in cortisol stress response and cortisol recovery in boys with oppositional defiant disorder/conduct disorder. *Psychoneuroendocrinology*. 2016;73:217-223. doi:10.1016/j.psyneuen.2016.08.007

39. Stadler C, Kroeger A, Weyers P, et al. Cortisol reactivity in boys with attention-deficit/hyperactivity disorder and disruptive behavior problems: the impact of callous unemotional traits. *Psychiatry Res*. 2011;187(1-2):204-209. doi:10.1016/j.psychres.2010.05.004

40. Frick PJ, White SF. Research Review: The importance of callous- unemotional traits for developmental models of aggressive and antisocial behavior. *J Child Psychol Psychiatry*. 2008;49(4):359-375. doi:10.1111/j.1469-7610.2007.01862.x

41. Kempes M, Matthys W, De Vries H, Van Engeland H. Reactive and proactive aggression in children. A review of theory, findings and the relevance for child and adolescent psychiatry. *Eur Child Adolesc Psychiatry*. 2005;14(1):11-19. doi:10.1007/s00787-005-0432-4

42. Craig SG, Moretti NGMM. A Systematic Review of Primary and Secondary Callous-Unemotional Traits and Psychopathy Variants in Youth. *Clin Child Fam Psychol Rev*. 2021;24(1):65-91. doi:10.1007/s10567-020-00329-x

43. Goulter N, Kimonis ER, Denson TF, Begg DP. Female primary and secondary psychopathic variants show distinct endocrine and psychophysiological profiles. *Psychoneuroendocrinology*. 2019;104:7-17. doi:10.1016/j.psyneuen.2019.02.011

44. Frick PJ, Ray J V, Thornton LC, Kahn RE. Can Callous-Unemotional Traits Enhance the Understanding, Diagnosis, and Treatment of Serious Conduct Problems in Children and Adolescents ? A Comprehensive Review. *Psychol Bull*. 2014;140(1):1-57. doi:10.1037/a0033076

45. Mackey S, Chaarani B, Kan KJ, et al. Brain regions related to impulsivity mediate the effects of early adversity on antisocial behavior. *Biol Psychiatry*. 2017;82(4):275-282. doi:10.1016/j.biopsych.2015.12.027

46. Schoorl J, Rijn S Van, Wied M De, van Goozen SHM, Swaab H. Neurobiological stress responses predict aggression in boys with oppositional defiant disorder/conduct disorder: a 1 - year follow - up intervention study. *Eur Child Adolesc Psychiatry*. 2017;26(7):805-813. doi:10.1007/s00787-017-0950-x

47. McCullough ME, Churchland PS, Mendez AJ. Problems with measuring peripheral oxytocin: Can the data on oxytocin and human behavior be trusted? *Neurosci Biobehav Rev*. 2013;37(8):1485-1492. doi:10.1016/j.neubiorev.2013.04.018

48. Martin J, Kagerbauer SM, Gempt J, Podtschaske A, Hapfelmeier A, Schneider G. Oxytocin levels in saliva correlate better than plasma levels with concentrations in the

cerebrospinal fluid of patients in neurocritical care. *J Neuroendocrinol*. 2018;30:e12596. doi:10.1111/jne.12596

49. Granger DA, Shirtcliff EA, Booth A, Kivlighan KT, Schwartz EB. The “trouble” with salivary testosterone. *Psychoneuroendocrinology*. 2004;29:1229-1240. doi:10.1016/j.psyneuen.2004.02.005

50. Schmalenberger KM, Tauseef HA, Barone JC, et al. How to study the menstrual cycle: Practical tools and recommendations. *Psychoneuroendocrinology*. 2021;123(104895). doi:10.1016/j.psyneuen.2020.104895

Table 1. Sample Characteristics, Comorbidities, Risk Factors, and Baseline Psychoneuroendocrine Levels in Participants With Conduct Disorder (CD) Compared to Healthy Controls (HCs)

	Females (n=186)		Males (n=104)				
	CD (n=82)	HCs (n=104)	CD (n=48)	HCs (n=56)	Group	Sex	Group x sex
	Mean (SD) or n (%)				*p*	*p*	*p*
Age (years)	14.83 (1.5)	14.58 (2.1)	14.54 (2.1)	14.88 (2.1)	.87	.98	.22
Estimated full-Scale IQ	97.32 (12.8)	104.33 (12.1)	101.21 (12.6)	105.75 (11.7)	<.001	.08	.42
Parental educational status	3.00 (1.0)	3.88 (1.1)	3.30 (0.7)	3.65 (0.9)	<.001	.78	.04
Body mass index	23.63 (6.5)	20.67 (3.9)	22.07 (4.0)	20.92 (3.6)	<.01	.27	.13
Start time Trier Social Stress Test (hh:mm)	15:08 (00:58)	15:16 (00:53)	15:07 (00:51)	14:56 (00:51)	.87	.12	.17
Pubertal status[a]					.13	<.001	
Early-pubertal	1 (1.2)	2 (1.9)	10 (20.8)	12 (21.4)			
Mid-pubertal	5 (6.1)	19 (18.3)	13 (27.1)	17 (30.4)			
Late-pubertal	57 (69.5)	54 (51.9)	23 (47.9)	24 (42.9)			
Post-pubertal	19 (23.2)	29 (27.9)	2 (4.2)	3 (5.4)			
Current smoking	51 (62.2)	7 (6.7)	25 (52.1)	9 (16.1)	<.001	.79	
Any medication[b]	48 (58.5)	12 (11.5)	10 (20.8)	2 (3.6)	<.001	<.001	
Neuroleptics	5 (6.1)	0 (0.0)	3 (6.3)	0 (0.0)	<.01	.92	
Stimulants	12 (14.6)	0 (0.0)	5 (10.4)	0 (0.0)	<.001	.57	
Non-stimulants	0 (0.0)	0 (0.0)	2 (4.2)	0 (0.0)	.12	.06	
SSRIs/antidepressants	5 (6.1)	0 (0.0)	2 (4.2)	0 (0.0)	<.01	.68	
Contraceptives	32 (47.1)	10 (13.5)	0 (0.0)	0 (0.0)	<.001		
Other	9 (11.0)	7 (6.0)	2 (4.2)	2 (3.6)	<.05	.35	
+LPE specifier	32 (39.0)	14 (13.5)	28 (58.3)	17 (30.4)	<.001	<.01	
Comorbidities							
Lifetime ADHD	31 (37.8)	0 (0.0)	28 (58.3)	0 (0.0)	<.001	.04	
Lifetime substance use disorder	18 (22.0)	0 (0.0)	14 (29.2)	1 (1.8)	<.001	.22	
Lifetime anxiety disorder[c]	18 (22.0)	2 (1.9)	17 (35.4)	2 (3.6)	<.001	.07	
Lifetime depressive disorder	35 (42.7)	4 (3.8)	9 (18.8)	0 (0.0)	<.001	<.01	
Lifetime PTSD	11 (13.4)	1 (1.0)	5 (10.4)	1 (1.8)	<.001	.82	
Pre- and postnatal risk factors							
Prenatal smoking	27 (40.9)	15 (16.1)	19 (48.7)	8 (15.7)	<.001	.54	
Prenatal violence	17 (25.4)	5 (5.4)	7 (18.4)	2 (2.4)	<.001	.42	
Adverse family situation					<.001	.99	
Either disharmony or isolation	25 (39.1)	16 (17.0)	11 (29.7)	12 (24.5)			
Both disharmony and isolation	20 (31.3)	3 (3.2)	11 (29.7)	1 (2.0)			
Number of traumatic events experienced[d]	2.78 (2.0)	1.08 (1.2)	2.13 (1.9)	1.46 (1.2)	<.001	.49	<.01
Baseline psychoneuroendocrine levels							

Cortisol (nmol/L)	2.91 (1.3)	2.64 (1.8)	3.22 (2.0)	3.35 (2.1)	.74	.02	.36
Testosterone (pg/ml)	39.64 (17.1)	34.30 (14.2)	60.40 (31.3)	58.12 (32.0)	.17	<.001	.59
Oxytocin (pg/ml)	1.37 (0.5)	1.39 (0.7)	1.07 (0.3)	1.27 (0.6)	.20	.02	.34
Psychological stress	1.51 (2.5)	1.21 (2.0)	1.43 (1.7)	0.94 (1.7)	.11	.48	.69

Note. Participants were assessed across five European sites (for details see Participants).

[a]For definition of pubertal categories, see Method.

[b]Non-stimulants include atomoxetine medication. 'Other' includes asthma medication, painkiller, or vitamin preparation. Participants may have been prescribed more than one medication category.

[c]Rates of lifetime anxiety disorder cover lifetime panic disorder, separation anxiety disorder, avoidant disorder, simple phobia, social phobia, agoraphobia, overanxious disorder, and generalized anxiety disorder.

[d]According to the PTSD section of the K-SADS-PL (for further details see Method). +LPE=with limited prosocial emotions specifier.

Table 2. Results of Hierarchical Linear Modeling of Psychoneuroendocrine Stress Responses in Participants With Conduct Disorder (CD) Compared to Healthy Controls

	Psychological stress				Cortisol				Testosterone				T/C ratio				Oxytocin			
	b	*SE*	β	*p*	*b*	*SE*	β	*p*	*b*	*SE*	β	*p*	*b*	*SE*	β	*p*	*b*	*SE*	β	*p*
OVERALL																				
Sex	0.12	0.11	0.06	.27	-0.07	0.13	-0.03	.58	-0.65	0.14	-0.31	<.001	0.02	0.13	0.01	.86	0.27	0.19	0.13	.16
Group	0.21	0.12	0.10	.09	-0.41	0.16	-0.21	<.01	-0.01	0.17	-0.01	.93	0.34	0.16	0.17	.03	-0.26	0.22	-0.13	.23
Time (poly 1)	-13.46	1.70	-0.28	<.001	0.04	1.23	<0.01	.97	2.18	0.74	0.07	<.01	0.42	1.68	0.01	.80	2.85	1.47	0.10	.05
Time (poly 2)	2.70	1.70	0.06	.11	-7.78	1.23	-0.20	<.001	-1.83	0.74	-0.06	.01	5.85	1.67	0.20	<.001	-3.96	1.47	-0.13	<.01
Group x sex	0.03	0.16	0.01	.87	0.05	0.19	0.02	.79	-0.07	0.20	-0.03	.75	-0.15	0.19	-0.07	.43	0.19	0.28	0.08	.51
Group x time (poly 1)	2.65	2.51	0.04	.29	-4.25	1.82	-0.07	.02	-2.24	1.08	-0.05	.04	3.11	2.49	0.07	.21	1.29	2.17	0.03	.55
Group x time (poly 2)	0.06	2.51	<0.01	.98	7.25	1.82	0.13	<.001	0.67	1.08	0.02	.54	-5.43	2.45	-0.12	.03	4.77	2.17	0.11	.03
Sex x time (poly 1)	-2.72	2.11	-0.05	.20	0.95	1.53	0.02	.54	-1.92	0.91	-0.05	.04	-1.52	2.10	-0.04	.47	-0.18	1.99	<0.01	.93
Sex x time (poly 2)	1.71	2.11	0.03	.42	-6.3	1.53	-0.13	<.001	-3.22	0.91	-0.09	<.001	6.09	2.08	0.17	<.01	1.53	1.99	0.04	.44
Group x sex x time (poly 1)	-2.10	3.14	-0.02	.50	0.24	2.27	<0.01	.92	2.07	1.35	0.04	.13	1.04	3.13	0.02	.74	-1.22	2.91	-0.02	.68
Group x sex x time (poly 2)	-1.28	3.14	-0.01	.68	5.30	2.27	0.07	.02	3.96	1.35	0.07	<.01	-4.50	3.09	-0.08	.15	-1.16	2.91	-0.02	.69
FEMALES																				
Group	0.23	0.10	0.11	.02	-0.32	0.16	-0.16	.05	0.11	0.16	0.07	.48	0.12	0.17	0.05	.49	0.01	0.26	<0.01	.97
Time (poly 1)	-16.18	1.30	-0.32	<.001	0.99	0.93	0.03	.29	0.25	0.56	0.01	.65	-1.14	1.36	-0.04	.40	2.67	1.37	0.09	.05
Time (poly 2)	4.41	1.30	0.09	<.01	-14.09	0.93	-0.38	<.001	-5.05	0.56	-0.21	<.001	11.95	1.34	0.38	<.001	-2.44	1.37	-0.08	.07
Group x time (poly 1)	0.55	1.95	0.01	.78	-4.01	1.40	-0.07	<.01	-0.17	0.85	<0.01	.84	4.17	2.05	0.09	.04	0.07	1.98	<0.01	.97
Group x time (poly 2)	-1.22	1.95	-0.02	.53	12.56	1.40	0.22	<.001	4.63	0.85	0.13	<.001	-9.93	2.03	-0.21	<.001	3.61	1.98	0.09	.07
MALES																				
Group	0.20	0.10	0.12	.04	-0.41	0.18	-0.20	.02	-0.12	0.18	-0.05	.52	0.42	0.16	0.23	.01	-0.31	0.24	-0.15	.19
Time (poly 1)	-13.46	1.59	-0.32	<.001	0.04	1.17	<0.01	.97	2.18	0.68	0.07	<.01	0.41	1.41	0.02	.77	2.85	1.44	0.10	.05
Time (poly 2)	2.70	1.59	0.06	.09	-7.78	1.17	-0.20	<.001	-1.83	0.68	-0.06	<.01	5.84	1.41	0.22	<.001	-3.96	1.44	-0.13	<.01
Group x time (poly 1)	2.65	2.34	0.04	.26	-4.25	1.72	-0.07	.01	-2.24	0.99	-0.05	.02	3.13	2.10	0.08	.14	1.29	2.12	0.03	.54
Group x time (poly 2)	0.06	2.34	<0.01	.98	7.25	1.72	0.13	<.001	0.67	0.99	0.01	.50	-5.40	2.07	-0.14	<.01	4.77	2.12	0.11	.02

Note. Time (poly 1) represents a linear (polynomial 1), and time (poly 2) a quadratic (polynomial 2) time effect. Reference category for gender=female, and for group=CD. SE=standard error of b, T/C ratio=testosterone/cortisol ratio.

Figure 1. Overview of Stress Test Procedure

Note. After a relaxation period in a comfortable room (Room A), the baseline assessment was taken, before participants entered a sparsely equipped experimental room (Room B). The Trier Social Stress Test (TSST) involved four components: task introduction, preparation time, speech task, and an age-adapted mental arithmetic task. For more information see the Methods section. CORT=cortisol; OXT=oxytocin; TEST=testosterone; VAS=Visual Analogue Scale.

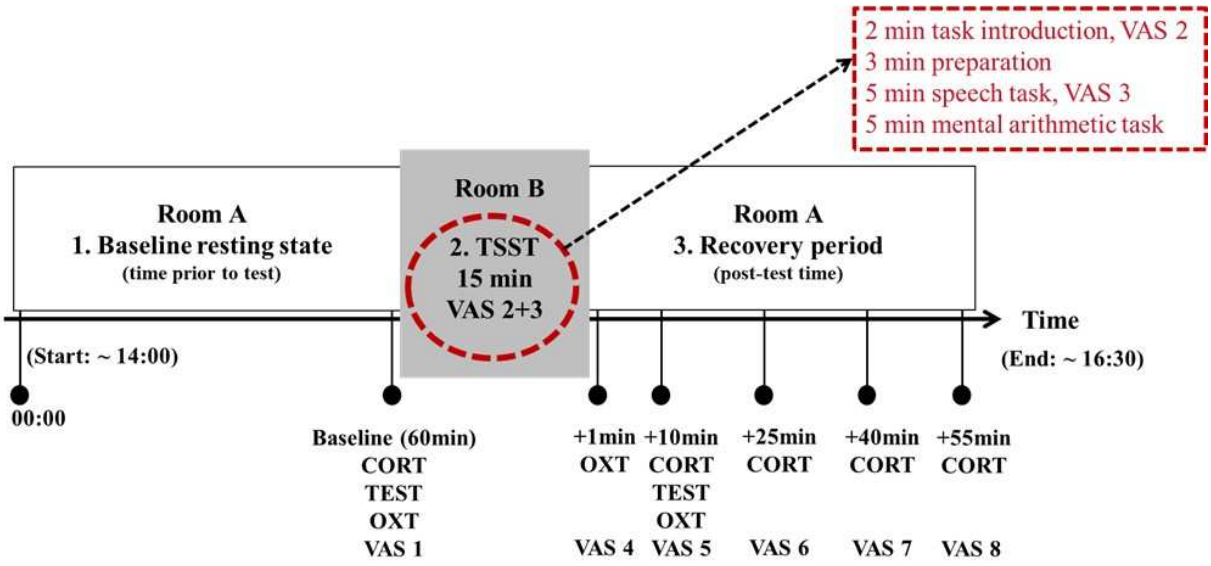
Figure 2. Psychoneuroendocrine stress response during the Trier Social Stress Test in participants with conduct disorder compared to healthy controls

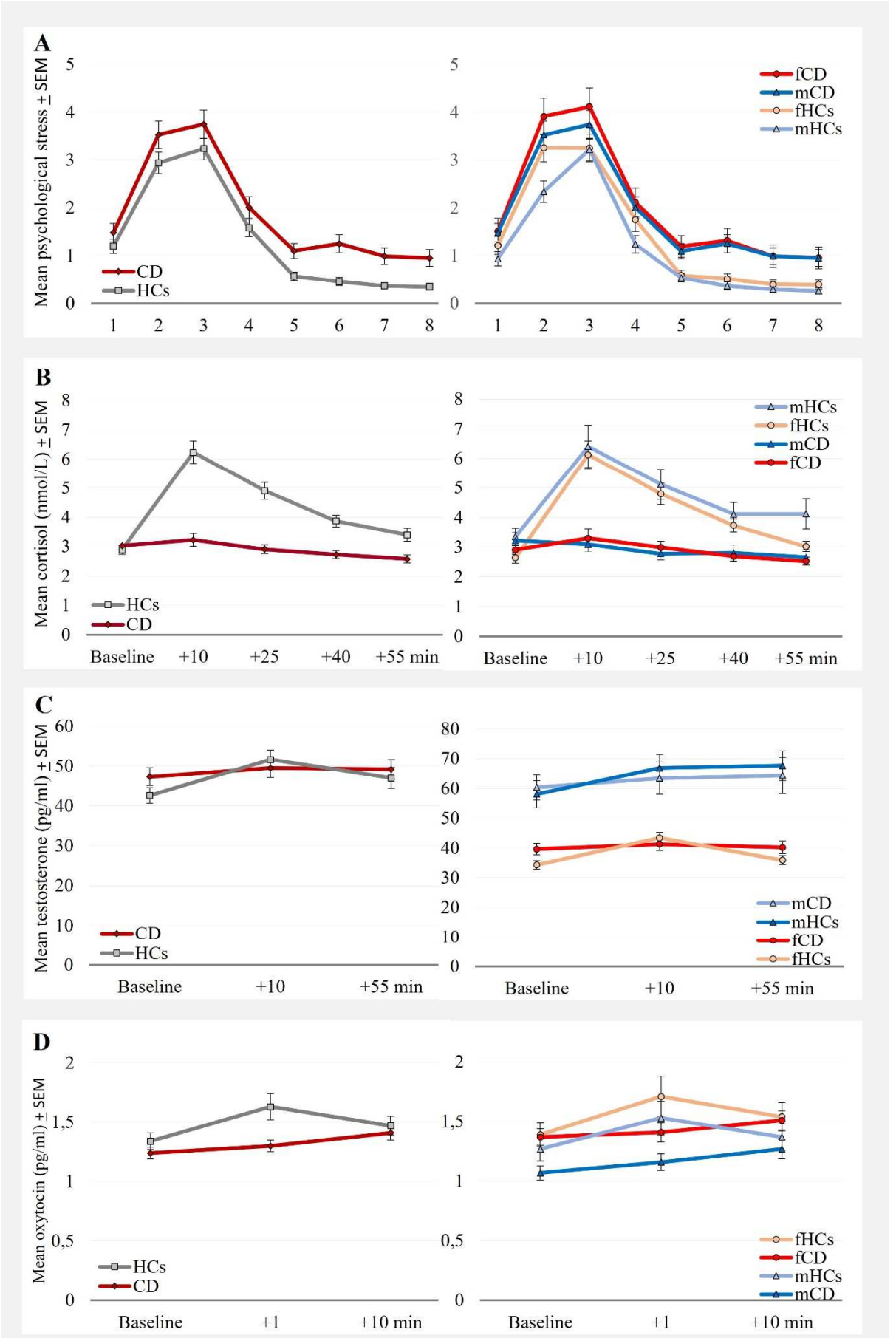
Note. Psychological Stress (A), Cortisol (B), Testosterone (C), and Oxytocin (D) responses in the overall (left) and sex-separated groups (right). CD=conduct disorder, fCD=female CD, fHCs=female HCs, HCs=healthy controls, mCD=male CD, mHCs=male HCs.

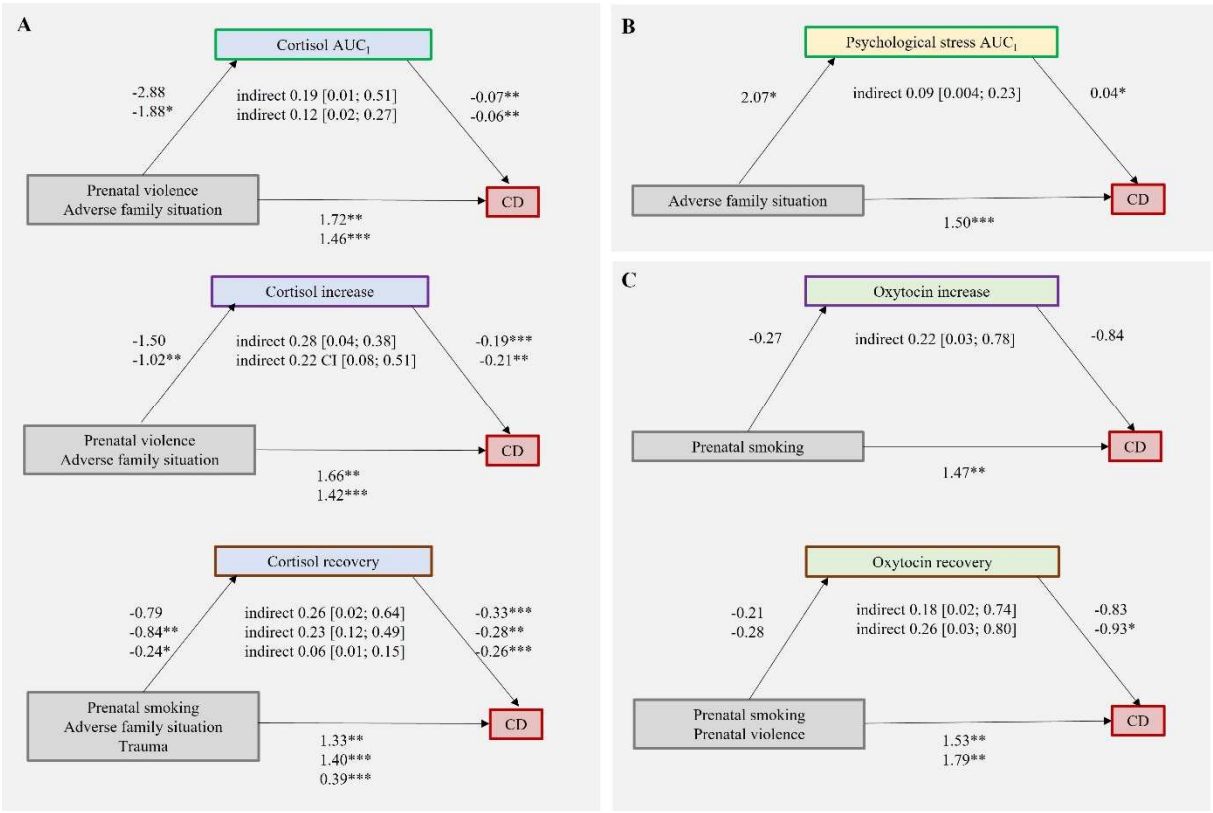
Figure 3. Psychoneuroendocrine stress response measures as mediators of the relationship between early environmental risk factors and conduct disorder

Note. Cortisol (A), psychological stress (B), and oxytocin (C) stress response measures during the Trier Social Stress Test as mediators. Numbers in brackets refer to 95% CIs. AUC_I=area under the curve with respect to increase, CD=conduct disorder.

*p<.05; **p<.01; ***p<.001.


2 min task introduction, VAS 2
3 min preparation
5 min speech task, VAS 3
5 min mental arithmetic task
Room A
1. Baseline resting state
(time prior to test)
Room B
2. TSST
15 min
VAS 2+3
Room A
3. Recovery period
(post-test time)
Time
(Start: ~ 14:00)
(End: ~ 16:30)
00:00
Baseline (60min)
CORT
TEST
OXT
VAS 1
+1min
OXT
VAS 4
+10min
CORT
TEST
OXT
VAS 5
+25min
CORT
VAS 6
+40min
CORT
VAS 7
+55min
CORT
VAS 8


A
Mean psychological stress ± SEM
CD
HCs
fCD
mCD
fHCs
mHCs
B
Mean cortisol (nmol/L) ± SEM
HCs
CD
mHCs
fHCs
mCD
fCD
Baseline
+10
+25
+40
+55 min
C
Mean testosterone (pg/ml) ± SEM
CD
HCs
mCD
mHCs
fCD
fHCs
Baseline
+10
+55 min
D
Mean oxytocin (pg/ml) ± SEM
HCs
CD
fHCs
fCD
mHCs
mCD
Baseline
+1
+10 min
Psychoneuroendocrinological assessment points


A
Cortisol AUC1
-2.88
-1.88*
indirect 0.19 [0.01; 0.51]
indirect 0.12 [0.02; 0.27]
-0.07**
-0.06**
Prenatal violence
Adverse family situation
CD
1.72**
1.46***
Cortisol increase
-1.50
-1.02**
indirect 0.28 [0.04; 0.38]
indirect 0.22 CI [0.08; 0.51]
-0.19***
-0.21**
Prenatal violence
Adverse family situation
CD
1.66**
1.42***
Cortisol recovery
-0.79
-0.84**
-0.24*
indirect 0.26 [0.02; 0.64]
indirect 0.23 [0.12; 0.49]
indirect 0.06 [0.01; 0.15]
-0.33***
-0.28**
-0.26***
Prenatal smoking
Adverse family situation
Trauma
CD
1.33**
1.40***
0.39***
B
Psychological stress AUC1
2.07*
indirect 0.09 [0.004; 0.23]
0.04*
Adverse family situation
CD
1.50***
C
Oxytocin increase
-0.27
indirect 0.22 [0.03; 0.78]
-0.84
Prenatal smoking
CD
1.47**
Oxytocin recovery
-0.21
-0.28
indirect 0.18 [0.02; 0.74]
indirect 0.26 [0.03; 0.80]
-0.83
-0.93*
Prenatal smoking
Prenatal violence
CD
1.53**
1.79**